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Evaluation of Iron Bioavailability from Bonito Dark Muscle Using Anemic Rats

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The bioavailability of iron from ferrous sulfate (FeII-S), heme iron prepared from hemoglobin (HIP), and bonito dark muscle (BDM) was assessed in anemic rats using a hemoglobin regeneration efficiency (HRE) method. Freeze-dried BDM (FD), boiled and freeze-dried BDM (B/FD), and boiled and smoke-dried BDM (B/SD) were used as BDM source. Rats were made anemic by feeding on an iron-deficient diet for 28 days. To replete their iron levels, anemic rats were then fed on a diet containing iron at a level of 17 ppm for 14 days. Rats receiving FeII-S gained significantly more weight and had greater food intake and higher HRE compared to the other four groups. The bioavailability of iron from HIP was poor compared with that from FeII-S and BDM. When the HRE of rats fed FeII-S was 100, that of rats fed BDF was ∼80. These results suggest that BMD is an effective dietary source of iron.

KEYWORDS: Iron bioavailability; hemoglobin regeneration efficiency; bonito dark muscle; rat

INTRODUCTION

The most common nutritional problem in both developing and industrialized countries is iron deficiency (*1*), which is usually attributable to inadequate iron intake, blood loss, or inadequate iron absorption. There are two forms of dietary iron: heme and non-heme. Heme iron is absorbed by the body more efficiently than non-heme iron (*2*). Heme iron is absorbed with the iron contained within hemoglobin (Hb) or myoglobin molecules, and its absorption is not affected by other foods in the diet (*3*). Therefore, heme iron is the best dietary source of iron and is found in meat, fish, and poultry.

The main muscle of most vertebrate fish is without pigment and essentially colorless, light muscle. Underneath the skin of many fish, however, is a layer of heavily pigmented, reddish brown muscle called dark muscle. The proportion of dark to light muscle varies with the activity of the fish. Active fishes such as bonito ("katsuo" in Japanese), mackerel, herring, and tuna have more dark muscle. In bonito, the ratio of dark muscle to total body muscle is ∼10−20%.

Dried and smoked bonito ("katsuobushi") has been used in Japan as a seasoning since ancient times. Katsuobushi is one of the most important materials in Japanese dishes. During the initial processing of katsuobushi, the dark muscle is cut off fresh bonitos to make fillets. The dark muscle is rich in myoglobin, which is an iron-containing monomeric heme protein found mainly in muscle tissue. Because there is little information on the bioavailability of iron in bonito dark muscle (BDM), this study was designed to evaluate this. We measured the hemoglobin regeneration efficiency (HRE) in anemic rats, which reflects the ability of anemic rats to utilize and retain dietary iron (*4*). This model was compared with other procedures used to assess the bioavailability of iron compounds in humans and was deemed to be a good predictor of iron compounds of high bioavailability (*5*).

MATERIALS AND METHODS

Test Materials. Freeze-dried bonito dark muscle (FD), boiled and freeze-dried bonito dark muscle (B/FD), and boiled and smoke-dried dark muscle (B/SD) were ground to a fine powder. Heme iron (HIP) was prepared according to the method of Tamura et al. (*6*). Briefly, citrate was added to fresh porcine blood to prevent coagulation; the blood was then centrifuged for blood cell collection. The cells were lysed by the addition of water and 5 mol/L NaOH solution to pH 9.0. The alkalized blood cells were hydrolyzed by alkaline protease (Alkalase, Novo Nordisk, A/S, Copenhagen, Denmark), and the fraction containing heme iron was collected by dialysis using an ultrafiltration membrane (SIP1013, fraction molecular weight 6000, Asahi Chemical Industries, Tokyo, Japan). Finally, this fraction was dried by a spray-dryer. The iron concentration in the HIP was 0.973 g/100 g, and no intact hemoglobin was detected in the heme iron. Protein content was determined according to the Kjeldahl method (*7*) using an N-to-protein conversion factor of 6.25. Total iron and non-heme iron in test materials were determined according to the method of Schricker (*8*). Heme iron was determined by the difference between total iron and non-heme iron. Protein and total iron contents and the ratio of heme iron to total iron in test materials are shown in **Table 1**.

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Table 1. Protein and Heme Iron Content in Test Materials

	protein (g/kg)	iron total (mg/g)	iron heme iron (%)
heme iron preparation (HIP)	831	9.73	100
freeze-dried bonito dark muscle (FD)	745	0.42	85
boiled and freeze-dried bonito dark muscle (B/FD)	805	0.42	86
boiled and smoke-dried bonito dark muscle (B/SD)	728	0.39	87

Table 2. Composition of Experimental Diets

	diet					
	FeD[a]	FeII-S[b]	HIP[c]	FD[d]	B/FD[e]	B/SD[f]
casein[g] (g/kg of diet)	250	250	248	195	198	181
corn oil (g/kg of diet)	50	50	50	50	50	50
Fe-free mineral mixture[h] (g/kg of diet)	40	40	40	40	40	40
vitamin mixture[h] (g/kg of diet)	10	10	10	10	10	10
ferrous sulfate[i] (g/kg of diet)		0.082				
heme iron preparation (g/kg of diet)			2			
FD (g/kg of diet)				55		
B/FD (g/kg of diet)					52	
B/SD (g/kg of diet)						69
sucrose (g/kg of diet)	650	650	650	650	650	650
Fe content[f] (mg/kg of diet)	1	17	17	17	18	17
Mg content[f] (mg/kg of diet)	572	572	572	570	578	571
Mn content[f] (mg/kg of diet)	53	53	53	53	53	53

[a] FeD, iron-deficient diet. [b] FeII-S, ferrous sulfate. [c] HIP, heme iron preparation. [d] FD, freeze-dried bonito dark muscle. [e] B/DF, boiled and freeze-dried dark muscle. [f] B/SD, boiled and smoke-dried dark muscle. [g] Edible lactic casein (80 mesh), purchased from New Zealand Dairy Board, Wellington, New Zealand. Casein contained 793 g of protein/kg. Iron content in casein was a trace amount. [h] Based on AIN-76 (AIN 1977). [i] The premixture consisted of 0.8 g of $FeSO_4 \cdot 7H_2O$ mixed with 99.2 g of sucrose. [j] The iron, magnesium, and manganese contents of the diets were measured by using atomic absorption spectrophotometry after wet-ashing with nitric acid/perchloric acid (3:1).

Animal Experiment This study was approved by the Laboratory Animal Care Committee of Ehime University, and the rats were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals of Ehime University.

Six-week-old male Wistar rats (Japan SLC, Hamamatsu, Japan) weighing 120−140 g were housed individually in screen-bottom aluminum cages in a room maintained at 23 ± 1 °C with an alternating cycle of light and dark of 12 h duration (light on, 7:00 a.m.−7:00 p.m.). The rats were acclimatized by feeding on a commercial solid diet (MF, Oriental Yeast Co., Osaka, Japan) for 7 days. After acclimatization, the rats were made anemic by feeding on a basal diet without any added iron (FeD diet, **Table 2**) and distilled deionized water for 28 days. The rats were divided into five groups ($n = 6$). Average body weight (122 ± 5 g), hemoglobin levels (4.5 ± 0.3 g/100 mL), and hematocrit (19.2 ± 0.4%) of different groups were similar when groups were initially fed the experimental diets. Ferrous sulfate (FeII-S), HIP, and various bonito dark muscle samples (FD, B/FD, and B/SD) were used as a source of iron and were added to the basal diet formulation to provide 17 mg of iron/kg of diet. All of the diets had the same protein concentration. The compositions of the experimental diets are given in **Table 2**.

The experimental diets and deionized water were offered to anemic rats ad libitum for 14 days. Body weight and food intake for each rat were recorded each morning before the diet was replaced. Feces from each rat were collected on the final 2 days of week 1 and the final 2 days of week 2 of the experimental period. Feces were freeze-dried, weighed, and milled. At the end of the experimental period, a blood sample from the neck of fed rats was collected into a blood collection tube (Vacutainer, Becton Dickinson, Franklin Lakes, NJ), which contained heparin as an anticoagulant.

The plasma was separated by centrifugation at 1400*g* at 4 °C for 15 min and was stored at −50 °C until analysis. After blood collection, the liver was perfused with cold saline (9 g of NaCl/L) to remove the blood. After perfusion, the liver, spleen, and heart were immediately removed, washed with cold saline, blotted dry using filter paper, and weighed; the liver was stored −50 °C until analysis.

Sampling and Analytical Procedures. To determine the levels of iron, manganese, and magnesium, the diet (∼500 mg) was wet-ashed in $HNO_3/HClO_4$ (3:1). To determine the level of iron, the powdered feces (∼70 mg), liver (∼3 g), and spleen were wet-ashed in $HNO_3/HClO_4$ (3:1). The concentration of magnesium was measured by atomic absorption spectrophotometry (AA 6400F, Shimadzu, Kyoto, Japan) after dilution in lanthanum chloride solution (5 g/L). The concentrations of iron and manganese were measured by atomic absorption spectrophotometry after dilution with deionized water. To determine the levels of iron, manganese, and magnesium, the standard addition method was used. The apparent absorption of iron was calculated as the difference between the dietary intake and fecal excretion of iron.

The hemoglobin concentration was measured by the cyanmethemoglobin method using a colorimetric hemoglobin assay kit (hemoglobin test, Wako Pure Chemical Industries, Osaka, Japan). Blood was obtained from the tail tip. To calculate total Hb content (grams) in the blood, the mass of blood was assumed to be 67 g/kg of body mass, and Hb was assumed to contain 3.35 mg of iron/g (*4*). HRE was calculated according to the method of Mahoney and Hendricks (*9*). The hematocrit was determined by centrifugation in a capillary tube system. The plasma iron concentration and unsaturated iron binding capacity (UIBC) were measured with a colorimetric hemoglobin assay kit (Fe-C test and UIBC test, Wako Pure Chemical Industries). Total iron binding capacity (TIBC) was calculated from the sam of plasma iron and UIBC.

In Vitro Digestion. A simulated physiological digestion of BDM was performed according to the method of Latunde-Dada and Neale (*10*). Briefly, BDM (∼1 g) was added to 30 mL of a 0.1 M HCl solution containing15 mg of pepsin from porcine stomach (EC 3.4.23.1; activity = 3100 units/mg of protein; Wako Pure Chemical Industries) and incubated at 37 °C for 1.5 h. After incubation, a 10 mL sample was taken and the pH was adjusted to 7.0 by gradual addition of solid sodium bicarbonate. After the neutralization procedure, a 5 mL sample was mixed with 5 mL of pancreatin-bile extract [4 g of pancreatin from hog pancreas (Wako Pure Chemical Industries) and 6.25 g of gall powder (Wako Pure Chemical Industries) dispersed in 250 mL of 0.1 M sodium bicarbonate], the pH was adjusted to 7.0, and the mixture was incubated at 37 °C for 1 h. Triplicates of 1 mL of digest at the different digestion stages were centrifuged at 8000*g* for 15 min. Supernatant was aliquoted into 5 mL tubes and dried. After drying, the residue was ashed at 600 °C for 6 h. After ashing, ash was dissolved in 1 mL of 1 M HCl, and the iron content was determined according to the *o*-phenanthroline method (*11*).

Statistical Analysis. Data from the animal study are expressed as means ± SEM. The statistical significance of a difference among groups was evaluated with one-way analysis of variance followed by Duncan's new multiple-range test using the Super ANOVA statistical software package (Abacus Concepts Inc., Berkeley, CA). The significance of relationships between variables was established by linear regression analysis with StatView (Abacus Concepts Inc.). Differences were considered to be significant at $P < 0.05$.

RESULTS

Animal Experiment. Body weight gain and food intake of rats fed the FeII-S diet were significantly greater than those of rats fed the other experimental diets (**Table 3**). The maximum apparent iron absorption was also observed in rats fed the FeII-S diet. Apparent iron absorption of rats fed the FD, B/FD, or B/SD diet was significantly higher than that of rats fed the HIP diet. Apparent iron absorption at week 1 was not significantly different among rats fed the FD, B/FD, or B/SD diet, but at week 2, absorption was at its highest in rats fed the FD diet,

Table 3. Food Intake, Body Weight Gain, Apparent Iron Absorption, Iron Content of Liver and Spleen, and Hemoglobin Regeneration Efficiency of $FeSO_4$, Heme Iron Preparation, and Bonito Dark Muscle Fed to Anemic Rats

	diet				
	FeII-S[a]	HIP[b]	FD[c]	B/FD[d]	B/SD[e]
food intake (g/14 days)	252 ± 11c	164 ± 3a	205 ± 9b	215 ± 5b	210 ± 6b
iron intake (mg/14 days)	4284 ± 187c	2788 ± 51a	3485 ± 153b	3655 ± 85b	3570 ± 102b
body weight gain (g/14 days)	105 ± 4c	45 ± 2a	82 ± 5b	85 ± 5b	80 ± 3b
apparent iron absorption (%)					
week 1	72.5 ± 1.9c	36.8 ± 3.1a	62.2 ± 1.3b	57.0 ± 2.4b	61.0 ± 1.5b
week 2	76.6 ± 2.6d	38.0 ± 1.6a	65.0 ± 2.3c	55.6 ± 1.9b	60.2 ± 1.0bc
organ weight (g/100 g of body weight)					
liver	4.95 ± 0.10c	3.59 ± 0.15a	3.96 ± 0.04ab	4.46 ± 0.14bc	4.44 ± 0.16bc
spleen	0.35 ± 0.09	0.36 ± 0.02	0.38 ± 0.03	0.38 ± 0.03	0.35 ± 0.03
heart	0.51 ± 0.02a	0.80 ± 0.04b	0.62 ± 0.03a	0.60 ± 0.04a	0.59 ± 0.03a
iron content in organs (fg/tissue)					
liver	156 ± 10c	82 ± 3c	117 ± 8b	120 ± 5b	126 ± 6b
spleen	104 ± 11b	73 ± 5a	77 ± 5ab	87 ± 4ab	78 ± 6ab
iron in plasma (fg/100 mL)	50 ± 11	38 ± 5	33 ± 5	37 ± 5	32 ± 3
TIBC (fg/100 mL)[f]	1286 ± 18	1421 ± 38	1345 ± 65	1382 ± 45	1321 ± 39
hemoglobin (g/100 mL)	8.4 ± 0.2c	3.5 ± 0.2a	5.6 ± 0.2b	5.3 ± 0.3b	5.9 ± 0.3b
hematocrit (%)	30.2 ± 0.3c	15.5 ± 0.7a	22.9 ± 0.5b	21.3 ± 0.9b	22.8 ± 0.9b
HRE[g]	0.75 ± 0.02c	0.22 ± 0.07a	0.60 ± 0.03bc	0.58 ± 0.03b	0.62 ± 0.02bc

[a–e] See *b*, *c*, *d*, *e*, and *f* in **Table 2**. [f] Total iron binding capacity (TIBC) = plasma iron + unsaturated iron binding capacity (UIBC). [g] Hemoglobin regeneration efficiency.

followed by rats fed the B/BD diet. Liver weight was at its highest in rats fed the FeII-S diet and at its lowest in rats fed the HIP diet. There was no significant difference in liver weight among rats fed the FeII-S, FD, or B/SD diet. Heart weight, per 100 g of body weight, in rats fed the HIP diet was significantly higher than in rats fed the other experimental diets; spleen weight, per 100 g of body weight, was not significantly different among rats fed the different experimental diets. The iron content of the liver in rats fed the FD, B/FD, or B/SD diet was significantly lower than in rats fed the FeII-S diet but was significantly higher in rats fed the HIP diet. The iron content of the spleen was significantly lower in rats fed the HIP diet compared to rats fed the other experimental diets, and there was no significant difference in iron content among rats fed the FeII-S, FD, B/FD, and B/SD diets.

The iron content of the liver in rats fed the FD, B/FD, or B/SD diets was significantly lower than in rats fed the FeII-S diet but was significantly higher than in rats fed the HIP diet. The iron content of the spleen was significantly lower in rats fed the HIP diet than in rats fed the FeII-S diet, but there was no significant difference among rats fed the FeII-S, FD, B/FD, and B/SD diets. Plasma iron concentration and total iron binding capacity (TIBC) were not significantly different among rats fed the different experimental diets. Hemoglobin and hematocrit values of rats fed the FD, B/FD, or B/SD diets were significantly lower than those of rats fed the FeII-S diet but were significantly higher than those of rats fed the HIP diet. The HRE of rats fed the HIP diet was significantly lower than that of rats fed the other experimental diets. The HRE of rats fed the B/FD diet was significantly lower than that of rats fed the FeII-S diet, but there was no significant difference in the HRE of rats fed the FD, B/SD, and FeII-S diets. A highly significant correlation between HRE and the apparent iron absorption was observed ($r = 0.990$ and $p = 0.0012$ for week 1 and $r = 0.957$ and $p = 0.0105$ for week 2).

In Vitro Digestion. About two-thirds of the iron in FD, B/FD, and B/SD was solubilized at the stage of HCl–pepsin digestion, but the iron solubility of HIP was only 2.4%. After neutralization with $NaHCO_3$, the iron solubility from FeII-S was markedly decreased, but that from HIP was markedly increased. The iron solubility of FD, B/FD, and B/SD, after neutralization with $NaHCO_3$, decreased by only half (**Table 4**). At all stages of the in vitro digestion procedure, the iron solubility from B/FD was lower than the solubility from FD and B/SD.

Table 4. Percentage Iron Solubility from Bonito Dark Muscle during Simulated in Vitro Digestion

	% soluble iron from			
	HIP[a]	FD[b]	B/FD[c]	B/SD[d]
HCl + pepsin, 1.5 h	2.4 ± 0.6	77.1 ± 2.5	59.3 ± 2.7	71.4 ± 2.9
HCl + pepsin + $NaHCO_3$ (neutralization)	39.6 ± 2.8	30.7 ± 1.0	39.8 ± 1.0	42.8 ± 2.5
HCl + pepsin + $NaHCO_3$ + pancreatin–bile extract digested for 1 h	11.4 ± 1.7	14.9 ± 0.5	13.9 ± 1.1	32.4 ± 4.5

[a–d] See *c*, *d*, *e*, and *f* in **Table 2**.

DISCUSSION

The bioavailability of iron from HIP was poor compared with that from FeII-S, FD, B/FD, and B/SD. Heme iron is relatively well absorbed under all circumstances. Moreover, the absorption of heme iron is relatively independent of meal composition and little affected by the enhancers and inhibitors that alter non-heme iron absorption. Hemoglobin is a complex of globin and four ferroprotoporphyrin or heme moieties. Conrad et al. (*12*) showed that hemoglobin was degraded to heme and globin degradation products in the small intestinal lumen by intestinal enzymes. Globin degradation products were important in maintaining heme in a soluble state so that it was available for absorption. Hemoglobin iron is better absorbed than heme iron without the globin (*13*, *14*). In HIP, the globin was removed from hemoglobin. Therefore, the poor iron bioavailability of HIP may be due to the removal of globin. However, Tabayashi et al. (*15*) have reported that heme iron (Heme Ace, Asahi Food, Tokyo, Japan), prepared as HIP has been in this study, prevents iron-deficient anemia in pregnant women.

The iron-deficient rats had lighter body weights (*16*). Suzuki et al. (*17*) reported that male weanling rats fed an iron-deficient diet have decreased food intake, body weight gain, and food efficiency. Total hemoglobin in mammals is proportional to body weight. Body weight gain and food intake were signifi-

cantly higher in rats fed the FeII-S diet compared to rats fed the HIP, FD, B/FD, and B/SD diets. In this study, food intake was found to be positively and significantly correlated with apparent iron absorption ($r = 0.948$ and $p = 0.0497$ for week 1 and $r = 0.879$ and $p = 0.0498$ for week 2). Body weight gain was found to be positively and significantly correlated with Hb concentration ($r = 0.936$ and $p = 0.0192$), HRE ($r = 0.979$ and $p = 0.0037$), and iron concentration in the liver ($r = 0.973$ and $p = 0.0053$). However, the iron-deficient rats had greater absolute and relative wet heart weights, demonstrating the presence of cardiac hypertrophy (*16*). Also, increases in the wet heart weight and size of cardiac muscle cells were observed in anemic rats compared with controls (*18*). In this study, the relative heart weight in rats fed the HIP diet was significantly greater than in rats fed the FeII-S diet, but there were no significant differences among the relative heart weights in rats fed the FD, B/FD, B/SD, and FeII-S diets. The relative wet heart weight was found to be negatively and significantly correlated with hemoglobin concentration ($r = -0.921$ and $p = 0.0262$), HRE ($r = -0.993$ and $p = 0.0007$), and iron concentration in the liver ($r = -0.973$ and $p = 0.0052$).

Anemia is a useful index of the severity of iron deficiency, but the significant liabilities of iron deficiency are more related to a deficiency in tissue iron. Among the body organs, the liver and spleen usually have the highest iron concentration, followed by the kidney, heart, skeletal muscles, and brain, which contain only $^1/_2$ to $^1/_{10}$ of the levels in the liver and spleen (*19*). The liver iron concentration in rats fed the FD, B/FD, and B/SD diets was significantly lower than in rats fed the FeII-S diet; however, there was no significant difference in the spleen iron concentrations. Iron occurs in blood as hemoglobin in the erythrocytes and as transferrin in the plasma, in a ratio of nearly 1000:1 (*19*). The hemoglobin concentration in rats fed the FD, B/FD, and B/SD diets was significantly lower than in rats fed the FeII-S diet. The apparent iron absorption at weeks 1 and 2 in rats fed the FD, B/FD, and B/SD diets was significantly lower than in rats fed the FeII-S diet. The liver iron concentration was found to be positively and significantly correlated with the apparent iron absorption ($r = 0.980$ and $p = 0.0034$ for week 1 and $r = 0.990$ and $p = 0.0012$ for week 2). Also, hemoglobin concentration was found to be negatively and significantly correlated with the apparent iron absorption ($r = 0.938$ and $p = 0.0018$ for week 1 and $r = 0.954$ and $p = 0.0012$ for week 2).

The proteolytic digestion of myoglobin and hemoglobin results in the release of heme, which is maintained in a soluble form by globin degradation products so that it remains available for absorption. The enzymatic degradation of myoglobin and hemoglobin is an important step in the absorption of heme iron. The heme moiety remains covalently bound to the polypeptide chain, which enhances solubility in aqueous solutions at a wide range of pH values. Also, certain amino acids, peptides, and polypeptides derived from bonito dark muscle are involved in the solubility of heme iron. The absorption of fortification iron depends primarily on its solubility in gastric juice (*20*). In this study, the iron solubilities from FD, B/FD, and B/SD by HCl−pepsin digestion were 77.1, 59.3, and 71.4%, respectively. The iron solubility from B/FD by HCl−pepsin digestion was lower than that from FD. The HRE of rats fed the B/FD diet was slightly lower than that of rats fed the FD diet, but the difference was not significant. Jacobs and Greenman (*21*) have shown that the amount of soluble iron was reduced after peptic digestion of cooked steak and bacon. In this study, decreased iron solubility, due to boiling, has been attributed to the denaturation and precipitation of dark muscle hemoproteins, which could not be significantly resolubilized under acidic conditions occurring during peptic digestion. In addition, the possibility of reduced protein digestibility on boiling may lead to decreased peptide and amino acid released on early digestion**,** which might reduce the formation of low molecular weight iron chelates that are normally well absorbed.

Iron absorption occurs predominantly in the duodenum and upper jejunum (*22*). After neutralization using $NaHCO_3$, the percentage of iron in the digested solutions from FD, B/FD, and B/SD was 45.5, 39.8, and 42.8%, respectively. Latunde-Dada and Neale (*10*) reported that the iron solubility from pigeon meat after neutralization using $NaHCO_3$ was 37.4%, and rats given lower dietary iron levels absorbed and retained greater quantities of iron from pigeon meat. In this study, the HRE of rats fed HIP was 0.22, which was similar to results of Weintraub et al. (*23*) and Park et al. (*24*). Meat iron has been shown to be readily utilized (*25*). The HRE of anemic rats fed a diet with beef as the iron source was 0.51 (*26*). The HRE of BDM was not inferior to that of beef. The HRE of rats fed the FD and B/SD diets was not significantly different from the HRE of rats fed the FeII-S diet. This suggests that iron from BDM is efficiently absorbed and retained in anemic rats.

In summary, the present study suggests that bonito dark muscle is an effective dietary source of iron. Further research is required to apply bonito dark muscle to practical use as an iron fortifier.

ABBREVIATIONS USED

BDM, bonito dark muscle; B/FD, boiled and freeze-dried bonito dark muscle; B/SD, boiled and smoke-dried bonito dark muscle; FD, freeze-dried bonito dark muscle; FeII-S, ferrous sulfate; HIP, heme iron preparation; HRE, hemoglobin regeneration efficiency.

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